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RESPIRATORY REGULATION IN *BUFO ARENARUM* EGGS [1]

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The study of the respiratory response of whole homogenates and the use of 2,4-dinitrophenol (DNP) as an uncoupling agent of oxidative phosphorylation, have proven to be useful tools to shed some light on the problem of respiratory control during amphibian development. The well established fact that an enhancement of respiratory activity can be obtained by homogenization (Spiegelman and Steinbach, 1945; Gregg and Ray, 1957; Gregg, 1960) as well as by treatment with DNP (Gregg, 1960; Legname, 1968), indicates that the *respiratory potential* exceeds the *respiratory norm, i.e.,* the respiratory rate exhibited by intact embryos under normal conditions (Gregg, 1960). That is to say that the developing egg relies on a respiratory system which is more than adequate to support the rate of normal respiration.

No conclusive evidence is available, however, regarding the mechanism by which the respiratory norm, held below the respiratory potential, increases as a function of developmental age. This was explained by Spielgelman and Steinbach (1945) and also by Gregg and Ray (1957), as a consequence of structural changes occurring in the course of development, which allow previously separated enzymes to contact their substrates. Later on, Gregg (1960) assumed that increasing respiratory rates would be the result of a parallel increase in the rate at which ATP is metabolized in response to energy requirements. Thus, as has been found in other cell systems, any decrease of the ATP pool with a concurrent increase of ADP and inorganic phosphorous, would activate oxygen consumption until the ATP level is re-established.

However, some unexpected findings have also been reported. For example, the homogenates of *Rana pipiens* eggs, prepared before the onset of gastrulation, were found to respire at about the same rate as intact eggs, in contrast to the respiration response observed following homogenization of older embryos (Gregg, 1960). Further, Gregg (1960), in the same species, and Legname (1968), in *Bufo arenarum,* failed to raise the respiratory activity of egg homogenates by means of DNP, in contrast to the marked effect obtained by treatment of intact eggs.

Two hypotheses have been proposed by Gregg (1960) to account for these observations. The failure of homogenization to elevate the respiratory activity of the segmenting egg has been ascribed to the formation of some coat, probably of lipo-protein nature, around the respiratory particulates impairing the availability of respiratory substrates. As to the lack of effect of DNP on egg homogenates, Gregg (1960) suggests that it could be the result of an activation of ATPase following cell disruption. Since ADP and inorganic phosphorus, under these conditions, have already reached a maximum level, no DNP effect can be expected.

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The present study was undertaken as an experimental approach to investigate the mechanisms underlying these puzzling effects with special reference to the question of embryonic respiratory control.

Material and Methods

Bufo arenarum oocytes were obtained by injecting adult females with a suspension of homologous hypophysis preserved according to Pisanó (1956). Fertilization was performed *in vitro* and embryos were reared in 10% amphibian Ringer solution without bicarbonate at laboratory temperature. The jelly coat was eliminated with 2% thioglycollic acid neutralized with KOH, followed by repeated washings with Ringer solution. Embryonic ages were determined by using the chart of Del Conte and Sirlin (1951) for the development of this species.

Homogenization was performed in a glass homogenizer with a teflon pestle, operated in an up-down manner by hand. The degree of homogenization was determined by the number of strokes which, except otherwise stated, was always six. Suspension media are indicated in each step. Homogenization was carried out in an ice cold bath.

The oxygen uptake was determined by the direct Warburg method using 17 ml reaction flasks with sidearm and center wells. Carbon dioxide was absorbed with 20% KOH contained in the center wells. The flasks were shaken at 80 cycles per minute at an ampltidue of 7 cm. Readings were made at 15 minute intervals.

When necessary, the following concentrations of cofactors and substrate were added to the homogenates: 0.12 μM Cytochrome C, 1.3 μM nicotinamide adenine dinucleotide (NAD) and 25 μM potasium fumarate. Final concentration of 2,4-dinitrophenol was 1.5×10^{-4} M. The final volume of the system was always 3.0 ml.

In order to determine ATPase activity, aliquots of the homogenates were incubated according to Maruyama and Ishida (1954), using 0.15 M histidine-KCl buffer, instead of sucrose. The liberated inorganic phosphorous was evaluated according to Marsh's technique (1959), microadapted to estimate 0.1 gamma (Puszkin, 1969).

Results

Respiratory effects of homogenization

Since Spiegelman and Steinbach (1945) and Gregg (1960), have reported contradictory data regarding the effects of homogenization on the respiration of *Rana pipiens* pregastrular embryos, experiments have been carried out in order to clarify inconsistencies.

The suspension media used by the above mentioned authors, 0.66 M phosphate buffer and 0.01 M phosphate buffer in 0.065% NaCl, respectively, have been tested. Under our working conditions, the suspension media do not affect significantly the respiratory relation of homogenized (as compared to intact) eggs. Homogenated respiration remained below that of intact controls (Table I).

TABLE I

Respiration of homogenized and intact eggs in different salt solutions. [*Values are expressed as μl of oxygen taken up; intact eggs: 300 blastulae per flask; homogenates: 3 ml containing 300 blastulae (100 blastulae per ml).*]

Eggs	0.066 M phosphate buffer		0.01 M phosphate buffer in 0.065 % NaCl	
	15 min	30 min	15 min	30 min
Intact	6, 3	14, 1	6, 1	13, 8
Honogenized	3, 1	6, 5	3, 5	6, 0

The number of eggs is another factor which has also been considered. Spiegelman and Steinbach (1945) used concentrations calculated to obtain 5 mm readings with 10 minute intervals, while Gregg uses 50 eggs per flask. Under our working conditions, such concentrations would correspond to 400–500, and 100–150 blastulae per flask, respectively. Table II shows that the respiratory ratio between intact and homogenized eggs varies according to the number of embryos used. In fact, while 150 homogenized blastulae do not exhibit respiratory activity, the oxygen uptake determined by 300 homogenized blastulae is equivalent to about 50% of that registered for the same number of intact eggs. Values *above* intact controls were obtained when using 500 homogenized blastulae.

When similar experiments were carried out adding cofactors and a substrate of the tricarboxylic acid cycle to the medium (Table III), the respiration of homogenates always exceeded that of intact eggs, regardless of the number of embryos used.

Respiratory effects of DNP on the homogenates

The lack of respiratory effects of DNP on homogenized amphibian embryos reported by Gregg (1960) in *Rana pipiens* and by Legname (1968) in *Bufo arenarum* has been reviewed. DNP respiratory effect depends upon the mechanical treatment to which cells have been submitted during brei preparation. In fact, Table IV shows that when working with early cleavage stages, which exhibit a

TABLE II

Effect of the egg number on the respiration of homogenates. [*Values are expressed as μl of oxygen taken up by 3 ml of different concentration of homogenized blastulae; suspension medium: 0.1 M phosphate buffer at pH 7.0; intact eggs: 150, 300 and 450 blastulae per flask; final volume: 3 ml.*]

Number of eggs per ml	15 minutes		30 minutes	
	Intact	Homogenized	Intact	Homogenized
50	3.8	0.0	7.6	0.0
100	6.3	3.0	12.6	4.6
150	9.0	14.6	20.1	29.3

TABLE III

Respiration of homogenized eggs in the presence of exogenous sustrate and cofactors. [Values are expressed as μl of oxygen taken up by 3 ml of different concentration of homogenized blastulae supplemented with cytochrome C, NAD and postasium fumarate; suspension medium: 0.1 M phosphate buffer at pH 7.0; intact eggs: 150, 300 and 500 blastulae per flask; final volume: 3 ml.]

Number of eggs per ml	15 minutes		30 minutes	
	Intact	Homogenized	Intact	Homogenized
50	3.0	6.6	5.6	13.2
100	6.1	13.1	13.8	27.5
167	9.0	21.5	21.1	46.4

considerable cell size, and using a dense suspension medium, it is possible to regulate homogenization so as to obtain a cell-free medium that can be stimulated by DNP, while a more drastic homogenization increases the oxygen uptake, and

TABLE IV

Influence of the degree of homogenization on the respiration of homogenates in the presence of 2,4-dinitrophenol. [Values are expressed as μl of oxygen taken up by 3 ml of homogenized first cleavage eggs containing 167 eggs per ml, suspended in a very dense medium (sucrose 1.2 M); final pH 7.0]

Degree of homogenization (pestle strokes)	15 minutes			30 minutes		
	Control	DNP	Δ%	Control	DNP	Δ%
2	8.2	15.0	82	18.0	32.1	76
10	14.7	14.7	0.0	32.0	30.0	−6

eliminates the effect of the uncoupling agent. Similarly, on very gently homogenized uncleaved eggs, DNP determines a respiratory increment similar to that detected on intact eggs (Table V).

TABLE V

Respiratory effects of 2,4-dinitrophenol on homogenized and intact eggs. [Values are expressed as μl of oxygen taken up by 3 ml of homogenized uncleaved eggs containing 167 eggs per ml, suspended in a dense medium (sucrose 1.2 M); final pH 7.0, degree of homogenization: One pestle stroke; intact: 500 uncleaved eggs per flask in 10% Ringer's solution, final volume 3 ml.]

Eggs	15 minutes			30 minutes		
	Control	DNP	Δ%	Control	DNP	Δ%
Intact	7.6	39.5	420	14.8	66.6	463
Homogenized	6.0	31.2	420	18.0	55.0	206

Mechanisms involved in the respiratory increment

In order to explain the mechanisms involved in the respiratory increment determined by homogenization, Gregg's hypothesis ascribing this effect to the stimulation of ovular ATPases resulting from cell disruption has been evaluated.

For this purpose, aliquots were taken from egg batches homogenized with variable intensity, in order to determine the oxygen uptake and the liberated inorganic phosphorous. Table VI shows that both oxygen consumption and

TABLE VI

Effect of homogenization on oxygen uptake and ATPase activity. [Oxygen taken up and inorganic phosphorus liberated by 3 ml of homogenized first cleavage eggs (100 eggs per ml). Homogenates were supplement with citochrome C, NAD and potassium fumarate; suspension media: Histidine-KCl buffer at pH 7.0.]

Degree of homogenization (pestle strokes)	Oxygen (μl)		P_i (μM)
	15 min	30 min	
1	6.6	13.2	0.33
3	9.0	18.1	0.45
6	11.0	21.1	0.57

ATPase activity, expressed as liberated inorganic phosphorus, increase with a marked parallelism according to the degree of homogenization.

On the other hand, Table VII shows that the respiratory increment determined by homogenization disappears after the addition of an ATPase inhibitor

TABLE VII

Effects of NaF, ADP and ATP on the respiration of homogenized eggs. [Values are expressed as μl of oxygen taken up by 3 ml of homogenized blastulae (100 eggs per ml) supplemented with citochrome C, NAD and potassium fumarate; ATP and ADP added: 50 μM; NaF: final concentration, 0.04 M; intacts: 300 blastulae per flask, final volume: 3 ml, suspension media: 0.1 M phosphate buffer at pH 7.0.]

Minutes	Intact	Homogenized				
		Control	NaF	ADP	ATP	ATP + NaF
15	4.6	10.4	4.5	18.5	10.9	4.9
30	8.3	17.1	9.1	33.4	18.9	9.8

such as sodium fluoride (NaF). This same table demonstrates that the respiration of homogenates, already above that of intact controls, can be further increased by adding ADP to the medium. The addition of ATP, on the contrary, does not affect the respiration of homogenates treated or not with NaF.

Discussion

The results described in the above section show that the effect of homogenization on the respiration of pregastrular embryos is independent of the suspension medium used, while the concentration of embryonic material appears to be of utmost significance in the relative rates of respiration of homogenized, as compared to intact, eggs.

These results also suggest that the dilution of the egg material, or of some of its components, would prevent the manifestation of a respiratory increment determined by homogenization. In this connection our results show that the respiration of homogenates may be made to duplicate that of the intact controls by adding cofactors as NAD and cytochrome C, and fumarate as a substrate, independently of the number of eggs used. In agreement with these results, isolated mitochondria of *Bufo arenarum* blastulae have been found to oxidize fumarate at a very high rate (Salomón de Legname, 1969).

The contradictory results obtained by Spiegelman and Steinbach and by Gregg, could be ascribed to the different concentrations of embryos used by these authors. The lack of effect reported by Gregg (1960) could be the consequence of a dilution of substrates and electron carriers, not occurring in Spiegelmann and Steinbach's experiences, which were performed using high concentrations of biological material.

As to the mechanisms determining an increment of oxidations by homogenization, Gregg (1960) also suggests that cell breakage may stimulate ATPase activity, thus resulting in an increase of ADP and inorganic phosphorus levels and, consequently, in an increment of respiration. Our results support this assumption (since a more drastic homogenization provokes a proportional increment on the respiratory and ATPase activity), and demonstrate in agreement with Gregg's hypothesis, that cell disruption determines an increment in the oxygen uptake through ATP hydrolysis.

On the other hand, the present results concerning the effects of concentration of embryos suggest an alternative to Gregg's hypothesis, which ascribed the failure of homogenization to increased respiration of very young embryos, to the formation of a membrane that would limit respiration to the capacity of certain substrates to penetrate lipoprotein barriers.

Confirmation of the present hypotheses could also account for the lack of respiratory effects of DNP in homogenized embryos reported by Gregg (1960) in *Rana pipiens* and by Legname (1968) in *Bufo arenarum*. In fact, since the activation of ovular ATPases seems to depend upon the mechanical treatment to which cells are submitted during brei preparations, the use of a very gentle homogenization which would not stimulate ATPases significantly would permit the action of the uncoupling agent. Such effect would disappear with a more severe homogenization. This hypothesis has been confirmed by using a very dense suspension medium and early-cleaving embryos, which exhibit a considerable cell size and permit a very gentle homogenization. Working with very carefully homogenized one-cell eggs, DNP determines a respiratory increment similar to that registered in unbroken controls.

Both factors, homogenization and DNP, stimulate oxygen uptake by altering the ATP:ADP ratio; therefore, the control of respiration during embryogenesis is likely to depend upon a similar mechanism in which ATPases would be most importantly involved.

The use of 0.04 M NaF as an ATPase inhibitor, show that the embryonic respiratory increment depends mostly on the concentration of ADP in the medium, while the role of ATP during the respiratory processes would be limited to that of an ADP donor. The slight increment in respiration detected after the addition of ATP, could be ascribed to the small amounts of ADP contaminating this compound.

Although NaF is not a specific ATPase inhibitor, no effect other than that can be ascribed to this compound under our working conditions. Its action would be limited to the annulment of the respiratory increment resulting from homogenization, while the oxygen uptake of intact eggs remain unaffected. In this connection, Barbieri and Valdez Toledo (1966) although working with lower concentrations, demonstrated that the respiration of *Bufo arenarum* blastulae is not affected by NaF. This effect had already been reported by Barth and Barth (1954) for *Rana pipiens*.

This assumption is further supported by the fact that NaF is known to act mainly at the level of the Embden-Meyerhof pathway, which accounts for only 20% carbohydrate degradation in *Bufo arenarum* blastulae. The remaining 80% is broken down via the pentose phosphate cycle (Salomón de Legname, Sanchez Riera and Sanchez, in preparation).

Summary

The more plausible hypotheses regarding the mechanism which controls amphibian embryonic respiration have been reviewed, providing some explanation for the contradictory respiratory ratios between intact and homogenized eggs reported by other investigators.

The concentration of embryonic material appears to be of the utmost significance in the relative rates of respiration of homogenized as compared to intact eggs. A close correspondence between increased oxygen uptake and the ATPase activity resulting from homogenization has been demonstrated. This would account for the lack of respiratory effect by 2,4-dinitrophenol which has been described in homogenized eggs of *Rana pipiens* and *Bufo arenarum*. Homogenization, as well as 2,4-dinitrophenol could stimulate oxygen uptake by altering the ATP/ADP ratio. By means of ATPase inhibition it has been also demonstrated that the respiration of homogenates is strongly affected by ADP while exogenous ATP is almost without effect.

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